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PRINCIPAL INVESTIGATOR: Kristina G. Flores

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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Duke University Medical Center
Durham, North Carolina 27710

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The adhesion properties of metastatic tumor cells and normal lymphocytes mediate their trafficking throughout the human body. The CD44 adhesion molecule is known to mediate the primary adhesion of lymphocytes to endothelial cells which is the initial step in the process of lymphocyte homing. Recently, isoforms of CD44 have been shown to be expressed on metastasizing tumor cells, which may allow metastatic tumor cells to mimic normal lymphocyte homing. Understanding the mechanisms by which CD44 mediates lymphocyte and tumor trafficking is critical for the development of immunotherapy designed to enhance effector lymphocyte trafficking to sites of tumor. The CD44 ligand hyaluronan has also been shown to promote angiogenesis, and tumor angiogenesis is predictive of tumor metastasis. We have investigated the mechanisms regulating CD44 isoform expression on endothelial cells and the role of CD44 in ligand binding and cellular trafficking of breast carcinoma cells and lymphocytes. The role of hyaluronan in angiogenesis was also investigated. Our data suggest that CD44 isoform expression is complex and varies depending on the activation and differentiation status of the cell by stimulation with specific reagents. We have also confirmed a role for hyaluronan in stimulating angiogenesis using an *in vivo* fibrin gel chamber assay in rats.

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Kristina A. Hess 9-14-98

PI - Signature Date

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Introduction

The original objectives of this grant were to investigate the mechanisms regulating isoform expression of the adhesion molecule CD44 on endothelial cells and the role of CD44 in ligand binding and cellular trafficking of breast carcinoma cells and lymphocytes. This study also included aims to investigate the mechanisms of CD44 in hyaluronan-induced angiogenesis. These studies were proposed to provide important insights into ways to increase the trafficking of leukocytes to sites of tumor and ways to prevent tumor cell metastasis and angiogenesis.

The following report will describe the extent to which the objectives of the original proposal were accomplished. We were able to investigate all three aims of the original proposal according to the proposed methods. The results and discussion section will point out the important contributions of the this work and will include the technical difficulties associated with these studies. Unfortunately, laboratory funding for supplies and technical support of this grant expired after the first year and it was necessary for the laboratory to redirect its research in areas that were also supported by the Duke University Specialized Program of Research Excellence (SPORE) in breast cancer. Thus, this report will also include a **redirection of research focus.**

CD44

CD44 is a structurally and functionally diverse transmembrane glycoprotein which is differentially expressed on the surface of a variety of hematopoetic and non-hematopoetic cells. A soluble form of CD44 circulates in human plasma [1]. Human CD44 is part of a large family of isoforms generated by alternative splicing of at least 9 variant exons within the extracellular domain and 1 of 2 cytoplasmic domains [2]. The CD44 adhesion molecule mediates the primary adhesion of lymphocytes to endothelial cells lining the blood vessel wall - which is the initial step in lymphocyte homing. Isoforms of CD44 which mediate normal lymphocyte trafficking are also expressed on metastasizing tumor cells and thus may potentially mediate tumor cell metastasis. Furthermore, the CD44 molecule binds and facilitates degradation of the extracellular matrix component hyaluronan, and low molecular weight forms of hyaluronan have been reported to promote angiogenesis, which in tumors correlates with metastatic potential [3, 4].

CD44 was first implicated in lymphocyte homing in 1987 [5]. Guo et al (1994) showed that soluble CD44 interferes with lymphocyte migration in a mouse model [6].

Other evidence confirmed CD44-hyaluronate binding in mediating lymphocyte rolling on endothelial cells using a parallel plate flow chamber [7]. This study also found that CD44-bearing lymphoid cell lines which do not normally exhibit the rolling phenotype can be induced to roll by activation with phorbol ester, a T-cell activator known also to induce HA binding. This finding suggests that CD44 mechanisms for lymphocyte extravasation from the blood may be activation-dependent.

CD44 is Involved in Tumor Metastasis

In 1991, Gunthert et al. isolated new CD44 variants found in metastasizing rat pancreatic adenocarcinoma cell lines. These variants were not found in non-metastasizing clones of the same cell line. Overexpression of these variant CD44 molecules in non-metastasizing cell lines resulted in the conversion of these cell lines to a metastatic phenotype [8]. This study and others have led to the hypothesis that the adhesive properties acquired by tumor cells for dissemination may be similar to CD44-mediated properties developed by normal lymphocytes to reach sites of antigenic invasion.

Tumor metastasis is dependent on the formation of new blood vessels to provide tumor cells with blood nutrients and access to vessels and the lymphatics. The extent of this process (i.e. angiogenesis) is believed to correlate with the metastatic potential of breast tumors [9]. Recent studies have shown that the CD44 ligand, hyaluronan, contributes to angiogenesis in several models. Low molecular weight hyaluronate products promote angiogenesis in a chick chorioallantoic membrane model [10] and in a delayed revascularization model using skin autografts in rats [11]. Studies disagree on the ability of the CD44v8-10 variant to bind and degrade hyaluronate [12, 13] and a recent *in vitro* study of cell migration and angiogenesis reports that full length hyaluronate promotes migration but not necessarily capillary formation [14]. Therefore, studies which address the angiogenic effects of hyaluronate fragments of characterized molecular weight as well as the contribution of CD44 to angiogenesis are needed.

Original Hypothesis/Purpose

The original hypothesis of this proposal was that CD44 isoform expression varies depending on the activation and differentiation status of a cell. The ability of endothelial cells, leukocytes, and breast carcinoma cells to participate in cellular trafficking or to bind specific ligands is influenced by CD44 isoform expression. Metastasizing tumor cells may mimic normal lymphocyte migration by using CD44 to mediate specific adhesion steps in cellular trafficking. Studies to determine CD44 specific mechanisms regulating cellular

trafficking and ligand binding are essential to the development of immunotherapy regimens designed to redirect and enhance lymphocyte trafficking to sites of breast tumor.

The original objectives of this grant were to investigate:

1) The mechanisms regulating CD44 isoform expression on human primary endothelial cells, endothelial cell lines of varying activation and differentiation status, and normal human breast and breast carcinoma tissues will be determined using time/dose response curves to a panel of reagents analyzed by solution phase and *in situ* RT-PCR, immunohistochemistry, and flow cytometry.

2) The adhesion properties of CD44 ligands, leukocytes, and breast carcinoma cells to endothelial cells will be defined using novel endothelial, breast carcinoma and T-cell lines expressing defined CD44 isoforms under static and flow conditions.

3) The mechanisms by which the CD44 ligand, hyaluronan, promotes angiogenesis will be evaluated *in vivo* in rodents using fibrin gel chambers containing hyaluronan fragments, blocking monoclonal antibodies, or CD44 peptides.

Body

Results and Discussion

Aim 1: CD44 isoform expression was analyzed in ECRF-24 endothelial cells, SKBR3 breast carcinoma cells, Jurkat T-cells and in primary human umbilical vein endothelial cells (HUVECs). These studies were performed using time and dose response curves with reagents such as TNF (tumor necrosis factor), LPS, PdBu or the spent supernatant of SKBR3 cells and analyzed by flow cytometry or by RT-PCR followed by standard Southern blot with CD44-specific oligonucleotide probes. These procedures were performed to test aim 1 with the expectations that the isoform expression of the cells and tissues described would vary depending on the activation status of the cell or tissue.

Preliminary time and dose response studies revealed expression of high molecular weight CD44 isoforms induced by exposure of primary HUVECs to spent supernatant from SKBR3 breast carcinoma cells. Some of these isoforms are also expressed constitutively by the EC-RF24 immortalized (and presumably activated) HUVEC line. EC-RF24 cells also induced a high molecular weight CD44 isoform upon incubation with 100μm PdBu. Flow cytometry experiments showed that the levels of other adhesion molecules, specifically ICAM-1, could be induced in Jurkat T-cells transfected with

specific CD44 isoforms. These data suggest that CD44 isoform expression is dependent on the activation or differentiation status of cells. CD44 isoform expression is thus a complex phenomenon and much additional work is needed to ascertain the signals for CD44 isoform expression and its consequences to the cell.

Aim 2: The adhesion properties of CD44 ligands, leukocytes and breast carcinoma cells to endothelial cells were tested in adhesion assays using endothelial, breast carcinoma and T-cell lines expressing defined CD44 isoforms. The above cells were transfected and selected to obtain those cells stably expressing defined CD44 isoforms. cDNAs for standard CD44 (exons 1-5, 15-17 and 18 or 19), CD44v8-10 (exons 8,9,10) or CD44v2-10 (exons 1,2,3,4,5,6,7,8,9,10) were used. A total of 14 exon specific cell lines were generated. Cells were tested in static adhesion assays whereby the binding affinity of calcein AM labeled cells to endothelial monolayers was quantitated using a fluorescent plate reader.

Static adhesion assays using fluorescently labeled CD44-transfected Jurkat T cells binding to monolayers of EC-RF24 endothelial cells showed a 780% increase in adhesion of the Jurkat cells expressing CD44v8-10 as compared to Jurkat cells expressing standard CD44 . This adhesion was not stable under high shear conditions generated by vigorous pipetting implicating an unstable interaction associated with primary adhesion. Other experiments showed that Jurkat T cells expressing CD44v8-10, an isoform expressed by activated normal T-cells, exhibited a 395% increase in adherence to HUVEC cells compared to standard CD44.

Studies using similar transfectants in the SKBR3 breast cancer cell line showed that SKBR3 cells transfected with vector alone adhered well to EC-RF24 cells. However, SKBR3 cells transfected with CD44v8-10 and CD44v2-10 showed a 26% and 45% increase, respectively, in adherence to EC-RF24 cells compared to SKBR3 transfected with vector alone. These studies suggested that CD44 isoform expression is important for T-cell and cancer cell adhesion to endothelial cells and should be investigated further.

Cell lines which exhibited strong, specific affinity in static assays were then tested in flow chamber assays designed to mimic the rolling and sticking of lymphocytes or breast cancer cells to endothelial monolayers under physiological conditions. In these dynamic assays, the binding affinity of Jurkat T-cell lines and SKBR3 breast carcinoma cell lines transfected with CD44 exons v8-10 to endothelial monolayers was tested in flow chambers under defined flow and shear conditions. These studies were performed in collaboration with Dr. Mark Dewhirst. According to static adhesion assays these cell were predicted to have some affinity in flow adhesion chambers but were predicted to lose adhesion under

stress [7]. We did not detect adherence of Jurkat T-cells or SKBR3 cells to endothelial monolayers. Although we expected to see a minimal level of adherence, this result confirmed static adhesion assays in which adhesion of cells was abrogated by high shear conditions.

The results of the static assays suggested that isoform expression did affect adherence of breast cancer cells to endothelial monolayers. However, followup studies in the flow chamber and subsequent repeats of the static adhesion assays led us to the conclusion that expression of certain CD44 isoforms increased homotypic adherence of transfected cells to each other rather than a greater adherence of single cells to the monolayer. This result was complicated by the observation that confluent cells do not necessarily cover the entire well. Consequently, the bottom of the well contains bare sites where fluorescent cells can adhere and contibute to fluorescent measurements. However, this result is still significant because homotypic adherence may affect metastatic potential by influencing the adherence of individual cells to the tumor mass. Flow chamber assays could not be used to further this investigation considering that adherence of cells could not be detected under the conditions used.

Aim 3: The mechanisms by which the CD44 ligand, hyaluronan, promotes angiogenesis was tested *in vivo* in rats using fibrin gel chambers containing low molecular weight hyaluronan fragments or full length hyaluronan which were sutured subcutaneously. This procedure was initially complicated by technical difficulties regarding the stability of the gel once inside the animal. However, the initial experiment showed an unexpected result in that full length hyaluronan promoted greater angiogenesis than hyaluronan fragments. This result was contradictory to earlier studies in which low molecular weight hyaluronan fragments promoted angiogenesis in chick chorioallantoic membrane models or in delayed revascularization models in rats. A critical technical problem which may have played a role in this result was the inability to maintain the stability of gels containing low molecular weight hyaluronan fragments. This problem has since been solved by refining the formation of the fibrin gels. These results did confirm the importance of hyaluronan in angiogenesis.

We decided next to use *in vitro* fibrin gel chambers. These chambers were created using a fibrin /thrombin gel solidified in a special co-culture insert of a 6 or 12 well plate (Costar). This assay was used to test the ability of endothelial cells to migrate through the fibrin gel towards a stimulus, specifically VEGF, in media placed directly below the gel. This assay also utilized CD44 antibodies to block migration of cells through the gel.

Our studies using the *in vitro* fibrin gel chambers revealed no detectable difference when CD44 antibodies were used to inhibit the migration of endothelial cells through a fibrin gel matrix. At this time, laboratory funding for this work expired and it was necessary to redirect the laboratory's focus of research.

Recommendations Regarding Statement of Work

The three aims of this grant were investigated using the proposed methods described in the original proposal. These studies provided valuable insights into CD44 isoform expression and its effects on cell to cell adhesion and confirmed the importance of CD44/hyaluronan in angiogenesis. The lack of appropriate funding and the time constraints in working out technical difficulties promoted a necessary and unavoidable shift in our research focus. Research is currently directed at investigating the function of the BRCA2 breast cancer susceptibility gene using T-cell receptor rearrangement in the thymus as a model. The remainder of this report will therefore focus on experiments and data regarding BRCA2 and will include and new statement of work for the remaining two years of this grant.

Redirection of Research Focus

BRCA2 Structure/Expression

BRCA2 was identified in 1995 by positional cloning [15]. Over 70 different mutations in BRCA2 occur in 45 percent of familial breast cancer cases. A high frequency of BRCA2 mutations are also found in families with increased incidence of pancreas, prostate and colon carcinomas and in male breast carcinomas [16]. The BRCA2 gene contains 27 exons, encoding a large protein with an estimated molecular weight of 384 kDa and has no significant homology to other known proteins. Exon 11, which at 5kb encodes almost half of the BRCA2 protein, contains 8 copies of a 30-80 amino acid domain [15, 17]. These 30-80 aa domains, called BRC repeats, are highly conserved between rat, mouse and human. These BRC repeats were recently found to mediate binding of BRCA2 to MmRAD51, the murine homologue of the RecA repair protein in bacteria, suggesting a possible role for BRCA2 in DNA repair [18, 19].

BRCA2 expression patterns reveal that although BRCA2 is widely transcribed, it is expressed at relatively low levels in most tissues. BRCA2 is only moderately expressed in normal adult breast tissue and in breast carcinoma. However, BRCA2 is expressed at high levels in breast terminal end buds during puberty and in differentiating alveoli during puberty [16]. **Interestingly, BRCA2 is highly expressed in normal ovary,**

testis and thymus, tissues with high levels of proliferation and differentiation.

BRCA2 is regulated during proliferation and differentiation. Rajan et al. (1996) found that BRCA2 is upregulated in proliferating and differentiating mammary epithelial cells similar to BRCA1. Therefore, BRCA1 and BRCA2 may function in overlapping pathways. BRCA2 mRNA levels peak at the G1/S boundary of the cell cycle and are low during the Go and early G1. [16, 20, 21]. Gene targeted null mutations of the BRCA2 exons 10 and 11 show that BRCA2 is required for embryonic cellular proliferation in the mouse. Mutant embryos exhibit developmental deficiencies and die before embryonic day 9.5. Mice carrying a different mutation in the 5' region of exon 11 survived to adulthood. These mice had various deficiencies including abnormal differentiation of tissues and most died due to the development of **thymic lymphomas** which originated from the immature $CD4^+$ and $CD8^+$ double-positive thymocytes according to flow cytometric analysis. BRCA2 -/- embryonic fibroblasts from these mice were also found to have an **impaired ability to repair double stranded DNA breaks** after X-irradiation. These cells were also shown to over-express p21 and p53 confirming the results by Ludwig et al. (1997) which showed that a p53 null background decreases the severity of a BRCA2 (and BRCA1) null mutation in mice [18, 22].

Several studies link BRCA2 to a role in repairing double stranded DNA breaks and/or suggest an association of BRCA2 with proteins thought to be involved in DNA repair. The first of these studies by Sharan et al. showed that mouse BRCA2 interacts with MmRAD51, the homologue of the RecA repair protein in bacteria . Since homozygous mutant embryos of MmRad51 exhibit hypersensitivity to ionizing irradiation, BRCA2 mutant embryos were predicted to act similarly. As expected, BRCA2 mutant embryos E.3.5 exposed to γ -radiation showed a significant decrease in the number of trophoblast cells compared to wild type embryos [23]. At least one other group has confirmed the BRCA2-RAD51 interaction using a yeast-two-hybrid screen [24]. Other evidence includes the observation that cultured murine embryonic fibroblasts containing a homozygous truncation mutation in exon 11 are sensitive to DNA damaging agents such as UV light and mitomycin C. These cells also accumulated a high frequency of chromosomal abnormalities with successive passages [25]. These studies strongly suggest that BRCA2 plays a critical role in DNA repair, particularly, in the repair of double stranded DNA breaks. This function of BRCA2 is intriguing considering that BRCA2 is also highly

expressed in the thymus, an organ which relies on double stranded DNA break and repair to achieve T-cell receptor gene rearrangement, a defining step in thymocyte development.

Current studies involving BRCA2 have been difficult due to the complications of working with such a large protein coupled with its low expression in most tissues including normal and neoplastic breast tissue. However, BRCA2 is highly expressed in the thymus, an organ with high levels of ongoing proliferation, differentiation, and DNA breakage and repair. These characteristics of the thymus are remarkably similar to the proposed functions of BRCA2. Therefore, the role of BRCA2 in the thymus is of great interest and importance. In addition, the thymus is a primary organ of the immune system as a site where T-cells rearrange their T-cell receptors and mature into functional cells. Little is known about why the adult thymus has a decreased ability to produce mature T-cells and this has adverse implications for breast cancer patients who receive high dose chemotherapy. Therefore, using the thymus to study breast cancer is useful in two important ways: 1) It is a necessary model to study BRCA2 function. One cannot ignore the data which suggests that BRCA2 has a specific role in the thymus perhaps as a factor for T-cell receptor rearrangement, and 2) Understanding the changes in immune function as the thymus ages (adult thymus vs. pediatric thymus) is important to patients with invasive breast cancer who have undergone high dose chemotherapy followed by bone marrow transplantation. These patients have a decreased ability to regenerate T-cells after chemotherapy. The complexity of the thymus is only now being appreciated. By understanding more about thymic signals and physiology we can potentially slow down or reverse thymic aging for the benefit of breast cancer patients, AIDS patients, the elderly or other individuals with immune deficiencies.

The Thymus and Thymocyte Development

The thymus is a primary organ of the immune system where T-cell development occurs. The thymus is a bilobed organ encapsulated by a thin layer of fibrous connective tissue which invaginates and separates each lobe into lobules. The lobules are further divided into an outer cortex and an inner medulla region. The septa that divides the lobules extends from the surface of the cortex to the medullary- cortical junctions and continues to widen into open spaces called the perivascular space (PVS). The PVS is distinct from the thymic epithelial-defined space and contains blood vessels and, particularly in older individuals, lymphocytes. Immature, proliferating T-cells reside in the thymus cortex. As they mature, T-cells migrate to the medulla to continue the maturation process. However, thymocytes are continually responding to signals that direct the cells to migrate, change cell

surface molecule expression, undergo T-cell receptor rearrangement , proliferate or apoptose as they traverse medullary-cortical boundaries. T-cells in both the cortex and the medulla are surrounded by a stromal network of cells including epithelial cells, dendritic cells, fibroblasts and macrophages. These cells also help support T-cell development via cytokines, cell surface adhesion molecules, and extracellular matrix components. T-cell development is tightly coupled with cell death or apoptosis to ensure that only functional T-cells survive the maturation process. Therefore, it is relatively clear why thymocyte development is an "expensive" process as only about 5% of thymocytes develop into functional T-cells and migrate to the periphery as effective immune cells [26, 27].

As the thymus ages it begins a process of involution. The true thymus tissue begins to atrophy and is replaced by adipose tissue while the perivascular space begins to widen and the connective tissue layer thickens. Involution and the widening of the PVS are thought to begin as soon as 1 year after birth and is complete by the 6th decade [28]. At the same time, T-cell growth and differentiation decrease and eventually cease. Although this usually causes no difficulties under normal conditions, loss of peripheral T-cells in a situation of thymic atrophy may potentially lead to immunodeficiencies and gaps in the T-cell repertoire. This situation may be harmful for adult cancer patients who receive high dose chemotherapy followed by bone marrow transplantation. The relatively minor T-cell losses seen in patients receiving standard dose chemotherapy may even affect the eligibility of patients to receive promising new immune based therapies. While high dose chemotherapy randomly kills healthy immune cells, the aged thymus has, in turn, little ability to regenerate functional T-cells. This study stresses the need for more research applied to basic immune function and is particularly applicable to breast cancer which is often treated using high dose chemotherapy .

T-cell Receptor Rearrangement

T-cell receptor rearrangement is a critical phase of thymocyte development . During maturation in the thymus, T-cells rearrange gene segments to form a functional T-cell receptor. The T-cell receptor is a heterodimer composed of either α and β chains or γ and δ chains. Gene segment rearrangement combined with other mechanisms for diversity allow up to 10^{18} combinations for the T-cell receptor. Gene segments to be recognized for rearrangement are flanked by recognition signal sequences (RSS). Each RSS is composed of conserved heptamer and nonamer motifs separated by nonconserved 12 or 23 base pair spacers. One gene segment containing a 12 base pair spacer can only rearrange with another gene segment containing a 23 base pair spacer. Recombination is mediated by numerous proteins, many of which are still unknown, which facilitate DNA cleavage,

synapsis and joining . Two proteins called recombinase activating gene 1 (Rag-1) and recombinase activating gene 2 (Rag-2) are absolutely essential for recombination to occur. These proteins recognize signal sequences then blunt-end cleave both strands of DNA. A 5' phosphorylated blunt end is generated at the signal sequence end while the gene segment end (coding end) forms a hairpin loop. Once the ends are generated, the coding ends are joined together with the addition or subtraction of nucleotides which contribute to diversity. The signal ends are then joined together precisely in a circular or linear complex [26, 29].

Assays for TCR Rearrangement

There are several different methods to analyze T-cell receptor rearrangement in DNA isolated from cells. Four distinct rearrangement assays are described briefly. (1) Southern Blot is a commonly used method for detecting TCR rearrangement. Genomic DNA is isolated from cells and digested with specific restriction enzymes which flank the gene segments of interest. Enzymatic digestion and gel electrophoresis of unrearranged DNA will produce a band of a unique size which contains all of the targeted gene segments and non-coding DNA. Alternatively DNA that has undergone rearrangement, will produce a different sized band based on elimination of restriciton sites and/or gene segements during the rearrangement process. 2). Other assays take advantage of Polymerase Chain Reaction (PCR) to identify rearrangement in a similar fashion. Generally speaking, PCR primers are generated which flank the gene segments of interest. Prior to gene rearrangement the distance between the primers is normally too large to detect by standard PCR due to intervening non coding DNA. However, if rearrangement has occurred the distance between the primers is decreased and is detectable by PCR. This assay is often followed by southern blot and detection using a specific probe. Southern Blot and PCR are particularly useful to detect rearrangement that has previously occurred. (3). Another assay, called ligation mediated PCR (LM-PCR) takes advantage of the generation of blunt DNA signal ends that are generated during rearrangement to detect either 5' or 3' breaks in the DNA that occur in real time. An asymmetrical linker is ligated to the blunt signal end. PCR primers specific to the asymmetrical linker and primers specific for sequences either upstream or downstream of the segment of interest are then used to amplify a product (See Figure 1). These products are transferred to membrane and probed for specificity. Differences in product sizes, as well as the specific primers used, help distinguish between 5' versus 3' breaks. This assay is different from the static PCR assay described above because the use of the asymmetrical linker targets DNA at the precise moment of double stranded DNA cleavage. This step distinguishes the DNA actively undergoing rearrangement from previously rearranged DNA molecules that do not contain breaks. (4) Finally, exogenous

rearrangement substrates can be introduced into cells and induced to rearrange by the action of native or exogenous RAG1 and RAG2. This can be performed in cells that do not normally rearrange their DNA, such as fibroblasts, by co-transfecting RAG-1, RAG-2 and a recombination substrate into the cell. Rearrangement of these substrates can be detected by PCR of the substrate DNA to detect a decrease in the size of a rearranged product or by using a recombination substrate that acts as a reporter gene after rearrangement has occurred.

Although these assays have been used previously in mouse cells, very little work has been done using human thymocytes. Duke University is one of few institutions where large quantities of human thymus tissue is readily accessible for research. Thus, we have been able to modify and optimize these assays for use in human cells. The proposed aim of this work is to study the involvement of BRCA2 in DNA repair processes using T-cell receptor rearrangement in the thymus as a model system.

Revised Technical Objectives:

1) **Develop dynamic *in vitro* assays to measure T-cell receptor rearrangement :**

a. Develop methods to detect DNA double stranded breaks as a function of T-cell receptor rearrangement by optimizing Ligation Mediated - PCR (LM-PCR) in primary human thymocytes.

b. Develop a recombination substrate assay to test T-cell receptor rearrangement in human cell lines in which BRCA2 levels can be modulated.

2) **Determine the effects of under- and over-expression of BRCA2 on T-cell receptor rearrangement as a model for general processes of DNA breakage and repair.**

a. Modulate BRCA2 using a panel of BRCA2 sense and antisense expression vectors and/or antisense oligos.

b. Measure effects of BRCA2 under- and over-expression on T-cell receptor rearrangement using LM-PCR or recombination substrate assays developed in objective 1.

Methods and Results

Objective 1a. Develop methods to detect DNA double stranded breaks as a function of T-cell receptor rearrangement by optimizing Ligation Mediated - PCR (LM-PCR) in primary human thymocytes.

Methods

Thymocytes are obtained from discarded thymus tissue by mechanical dissociation, washed and resuspended in RPMI +20% FCS. For studies in objective 2, thymocytes will be transfected with BRCA2 or control antisense oligonucleotides using Lipofectamine and cultured for 24 or 48 hours prior to analysis. Genomic DNA is isolated from thymocytes at 0 hours, 24 hours or 48 hours after transfection using a previously described method [30] for analysis by LM-PCR .

LM-PCR to detect soluble stranded DNA breaks was modified and optimized from previously described protocols [31]. We have chosen to analyze DNA breaks which occur 5' or 3' to the Dβ2.1 TCRβ chain locus since the human genome contain only 2 Dβ loci. This allows dectection of V-DJ and D-J breaks in 50% of thymocytes undergoing rearrangement.

An asymmetrical linker is generated by incubating 2 nmoles of Linker-A and Linker-B oliognucleotides in 100 ul of 250mM Tris (pH 8.0) for 5 minutes at 90° C then 5 minutes at 60°C. Genomic DNA (1-2ug) is ligated to 20 pmoles of asymmetrical linker for 12-16 hours at 16°C. The reaction is stopped by heating in a Tris, KCL, Triton X-100 based stop solution .

Serial 8 fold dilutions of linker ligated human DNA are made into mock linker ligated mouse DNA to keep the total amount of DNA constant for each sample. DSBs from each dilution are then detected by nested PCR (See Figure 1). PCR products are run on a 2% polyacrylamide gel and Southern blotted by standard methods. Specific bands corresponding to dsDNA breaks either 5' or 3' of the Dβ2.1 locus are detected using a ^{32}P labeled probe which spans the Dβ2.1 locus but which does not include primer regions.

LM-PCR Detects D-J and V-DJ Rearrangement in Thymocytes From Pediatric Patients. The LM-PCR assay optimized as described above was used to detect rearrangements occurring 5' or 3' to the Dβ 2.1 human locus. The Dβ probe detects a 503 bp band corresponding to a D-J rearrangement and a 422 bp band corresponding to a V-DJ rearrangement. The LM-PCR assay is able to detect rearrangement as it is occurring

in normal human pediatric thymocytes (See Figure 2). Note that control lymphocytes taken from spleen and lymph node do not contain Dβ rearrangement signals. Higher and lower molecular weight signals vary between samples and are most likely due to additional rearrangements occurring at other TCR loci. The rearrangement signals at bp 503 and 422, indicative of TCR β chain rearrangement, are consistently present in pediatric thymocytes.

1b. Develop a recombination substrate assay to test T-cell receptor rearrangement in human cell lines in which BRCA2 levels can be modulated

Use of TCR Rearrangement Constructs in Cells in which BRCA2 levels can be Modulated Primary thymocytes are difficult to transfect with expression plasmids resulting in extremely low transfection efficiencies using these cells. It is relatively easy to transfect oligonuleotides into thymocytes but difficult to select and analyze transfected cells due to rapid cell death. Conversely, many cultured cell lines can be easily transfected with BRCA2 antisense plasmids, antisense oligos or adenovirus vectors with high transfection efficiencies and high cell viability. We will use a novel recombination substrate and Rag-1 and Rag-2 constructs obtained from Dr.Guillermo Taccioli to transfect 293T and breast cancer cells. This assay will enable us to determine the effects of modulation of BRCA2 on TCR-type rearrangement in these cells.

In Vitro Recombination Assays Using a Recombination Substrate. We are currently generating a novel recombination substrate using green fluorescent reporter protein to analyze double stranded DNA breaks in cultured cell lines. This recombination substrate, like many previously described, is comprised of a recombination signal sequence integrated between two segments of a reporter gene. The reporter gene containing the RSS is nonfunctional before recombination. When recombination occurs, the RSS is excised and the segments of the reporter gene are joined together to form a functional gene. Our recombination substrate is derived from insertion of the recombination signal sequences regulating recombination of the Dδ and Jδ loci of the D2J transgene (obtained from Dr. Mike Krangel) into the GFP reporter gene. To create a modified GFP containing a RSS, the GFP protein was first cleaved near the N-terminal region before its fluorophore containing barrel structure (See Figure 3). A unique SacI site was generated in the N-terminal region of the GFP molecule (between codons for amino acids 7 and 8) by site directed mutatgenesis. The RSS was generated by PCR using SacI terminal primers and was thus specifically inserted into the newly formed SacI site to generate the recombination substrate. The sequence of this substrate has been confirmed by DNA sequencing.

Construction of A Novel TCR Rearrangement Substrate To determine if the GFP protein could withstand the addition of nucleotides (due to junctional flexibility, TdT additions and nuclease activity) in the N terminal region, we added 12 additional nucleotides encoding 4 amino acids between the SacI site at bp 635. The additional nucleotides collectively termed the "Sac linker" were created by self-hybridization of a 16 bp oligo to form a ds DNA molecule with sticky Sac I ends and the NgoMI restriction enzyme site in the center. Constructs containing the Sac linker were identified by digestion with the NgoMI enzyme. The construct containing the Sac linker was transfected into Cos-7 cells and GFP function was measured by flow cytometry. Cells containing pEGFP modified with the Sac linker show strong fluorescence when measured by flow cytometry after a 48 hour transfection. This experiment shows that GFP is still functional after mutation with up to 12 additional nucleotides in the barrel region and therefore a GFP containing the RSS should function similarly after recombination and imprecise joining (See Figure 3).

Recombination Assays Using A Novel Recombinaiton Substrate The recombination substrate described above will be transfected into 293T and breast cancer cells which have been previously shown to be sufficient to induce TCR-type recombination of similarly designed substrates in fibroblasts and other non-lymphpocyte systems. After 48 hours, cells will be analyzed for recombination by two methods: First, low molecular weight DNA will be extracted from transfected cells and analyzed for the presence of correctly rearranged GFP by PCR using GFP-specific primers. Recombination will result in a band of 267bp versus 1200bp for unrearranged GFP. Relative numbers of rearranged molecules will be approximated using serial dilutions and PCR standards. Second, the numbers of cells with correctly rearranged recombination substrates and expressing fluorescent GFP will be measured by flow cytometry. We are beginning these experiments now and anticipate optimization of transfection and assay conditions to complete this objective within the next few months.

Objective 2: Determine the effects of under- and over-expression of BRCA2 on T-cell receptor rearrangement as a model for general processes of DNA breakage and repair:

a. Modulate BRCA2 using a panel of BRCA2 sense and antisense expression vectors and/or antisense oligos.

b. Measure effects of BRCA2 under- and over-expression on T-cell receptor rearrangement using LM-PCR or recombination substrate assays developed in objective 1.

BRCA2 Expression High levels of BRCA2 RNA were found to be associated with whole thymus vs. thymic stroma consisting of epithelial cells, fibroblasts, macrophages (See Figure 4). The majority of the BRCA2 RNA found in whole thymus was further localized to the thymocytes by study of RNA expression . This study suggests that thymocytes, in particular, express BRCA2 RNA versus other thymic cells including epithelial cells, macrophages and fibroblasts contained in the thymic stroma. No BRCA2 RNA is detectable in thymic stroma by Northern blot with equal loading. Preliminary studies to determine differences in BRCA2 levels in subsets of thymocytes are currently being performed. Using RT-PCR, we have determined that BRCA2 mRNA is expressed in thymocyte subsets in which β chain (CD4, CD8, CD3 triple negative thymocytes) and α chain rearrangement (CD4, CD8 positive thymocytes) occur. These studies also suggest that a role for BRCA2 in thymocytes rearranging their DNA is plausible. Additional experiments to confirm and quantitate BRCA2 levels in thymocytes compared with mature T-cells which have completed TCR rearrangement will also be performed.

BRCA2 Expression Constructs Our laboratory has previously generated a full length BRCA2 expression construct and several BRCA2 antisense expression vectors and oligonucleotides. Cos cells transfected with full length BRCA2 show a marked increase in protein by western blot and in RNA levels by Northern (Data not shown). In addition, full length sense and antisense BRCA2 was inserted into the PBI-Super plasmid (Clontech) for use in the Tet-Off Expression system (Clontech). This system allows expression in the absence of tetracycline in cells transfected with the tetracycline transcriptional activator (tTA). We are currently attempting to generate a full length BRCA2 construct in adenovirus which can be used in a greater number of cells to achieve maximum transfection efficiencies. This effort is complicated by the large size of BRCA2 cDNA which necessitates the use of adenovirus containing additional deletions. Other BRCA2 antisense constructs generated near the BRCA2 translational start site at 229 include BRCA2 bp (792-238), (bp 23-229) and (bp 369-23) in a eukaryotic expression vector. The eukaryotic vector used for BRCA2 antisense constructs contains a 5' GFP tag to monitor transfection efficiencies in cells. Oligonucleotides generated near the translational start site will be used to block BRCA2 expression in primary thymocytes because they can be transfected with relatively high efficiencies in these cells compared to expression vectors.

Each of these constructs will be tested for its ability to induce or to abrogate expression of BRCA2 (expression and antisense constructs, respectively). The constructs which work most efficiently to abrogate or increase BRCA2 expression will be utilized in subsequent studies which will test the effects of BRCA2 modulation on DNA repair processes using LM-PCR (for thymocytes transfected with antisense oligos) and recombination substrates. It is anticipated that these studies will provide important insights into mechanisms of BRCA2 function, which may result in preventative therapies useful for patients with germline BRCA2 mutations.

Summary

In this report, I have described progress made toward our original Technical Objectives in the role of CD44 variants in tumor cell trafficking and angiogenesis. Unfortunately, funding considerations prompted a redirection of research focus. I have presented preliminary data gathered from studies aimed at elucidating the mechanisms of BRCA2 function. Revised Technical Objectives and a Revised Statement of Work are appended.

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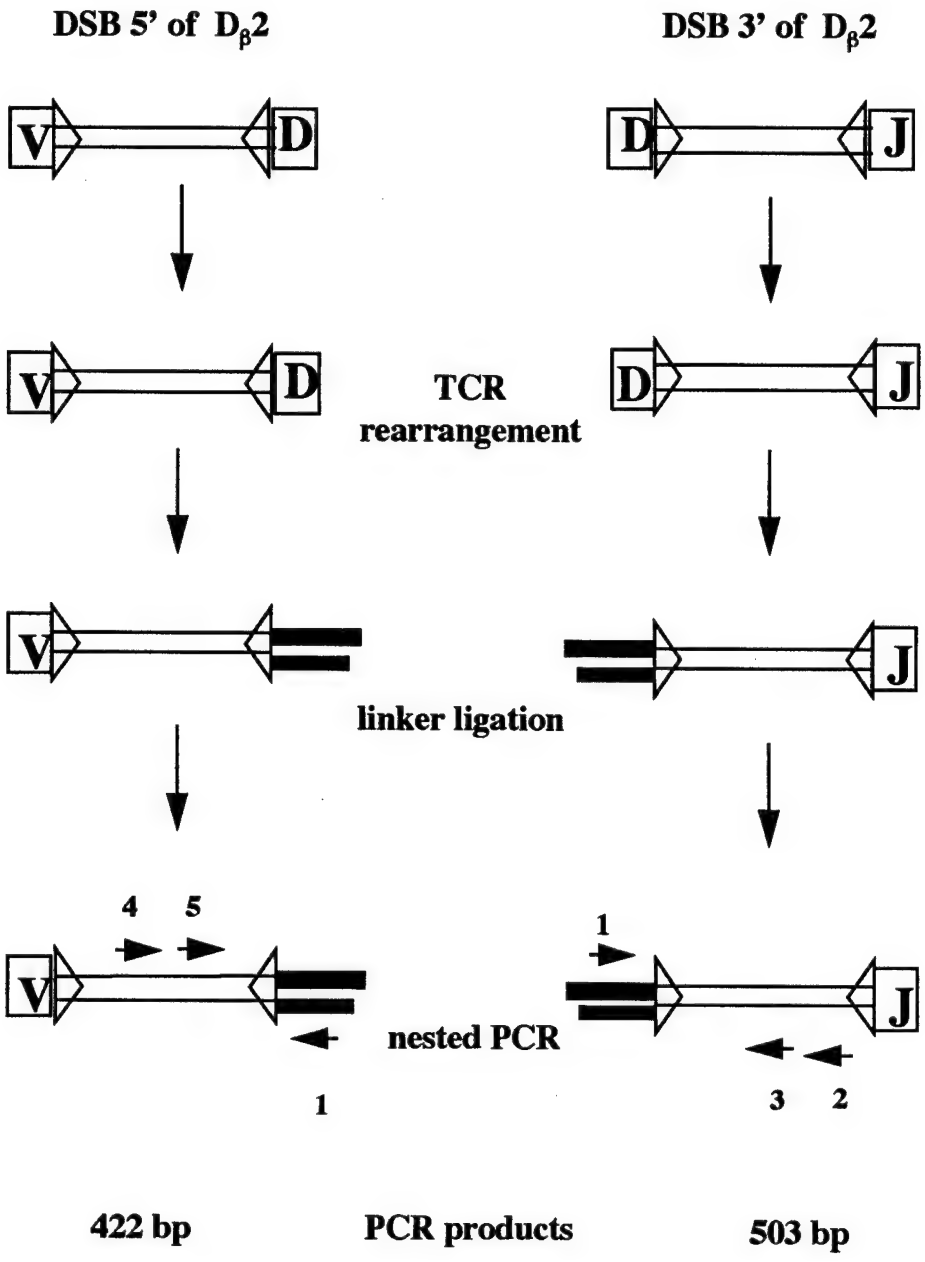


Figure 1. Ligation Mediated PCR. Genomic DNA from thymocytes is harvested and used to detect double stranded DNA breaks occurring 3' or 5' of the Dβ locus during VDJ rearrangement. An asymmetrical linker is then ligated to the blunt ended DNA break. Primers specific for the the linker (primer 1) and Dβ upstream primers (primers 4, 5) or Dβ downstream primers (primers 2, 3) are used in nested PCR reactions to obtain PCR products with a specific length.

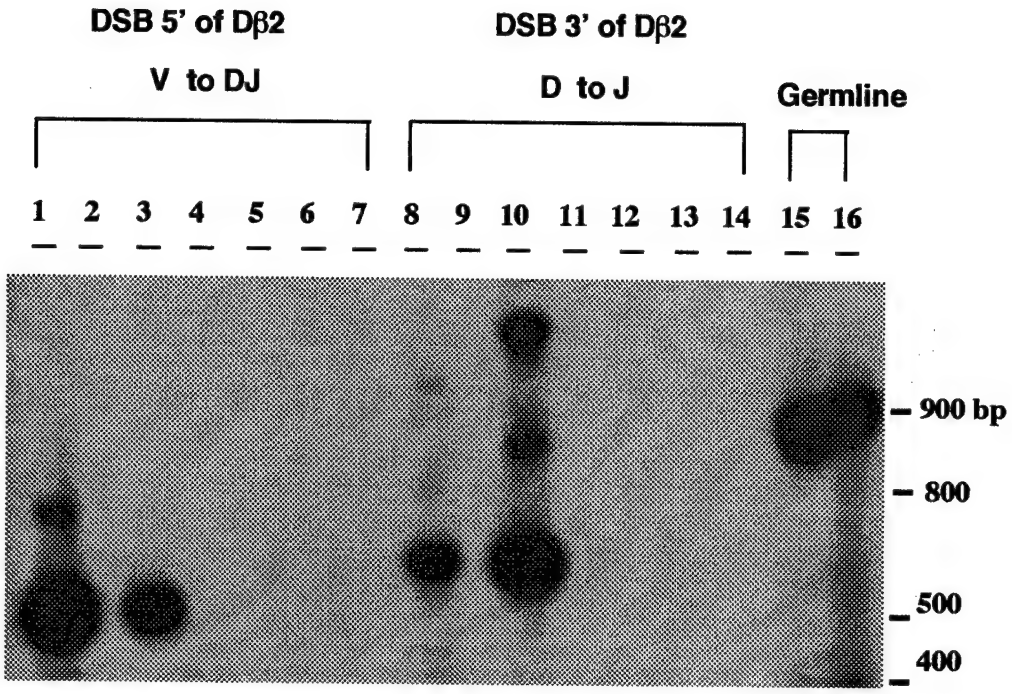
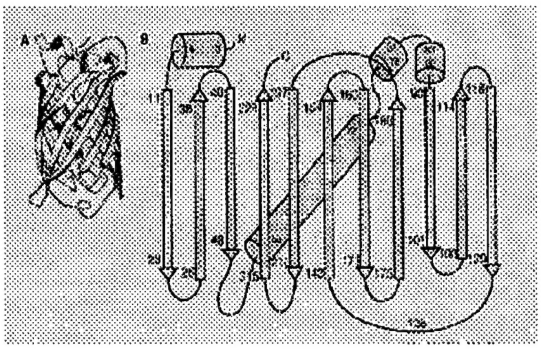
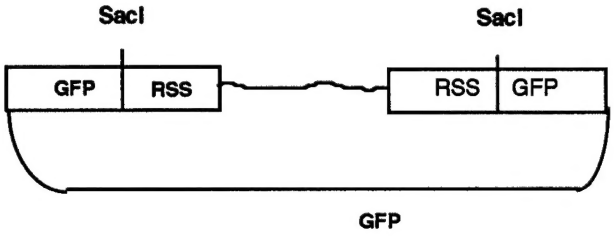


Figure 2. LM-PCR detects rearrangement signals from pediatric thymocytes. Bands at 422bp and 503 bp represent double stranded DNA breaks 5' or 3' to the Dβ2 locus respectively. Lanes 1 and 8 represent pediatric thymus A using forward and linker primers. Lanes 3 and 10 represent pediatric thymus B using reverse and linker primers. Lanes 15 and 16 demonstrate amplification of genomic DNA using inner nest primers for thymus A and outer nest primers for a spleen control sample. Lanes which do not have specific rearrangement signals are as follows: lane s 5 and 12 represent bacterial DNA, lanes 2,4,6,9,11,13 contain corresponding DNA samples which were not ligated with linker DNA. Lanes 7 and14 are PCR controls.

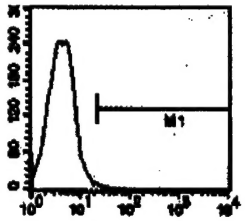
A.



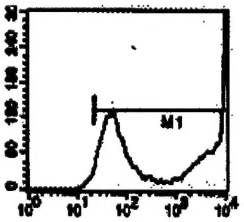
B.



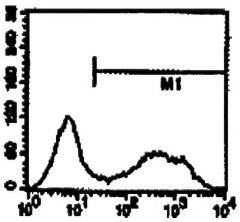
C.



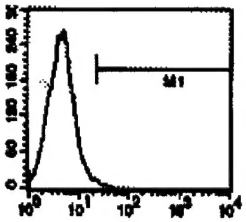
control



wt EGFP



EGFP-Sac linker



EGFP-RSS

FIGURE 3. Panel A depicts the structure of GFP. Arrows indicate the site for rearrangement substrate insertion. Panel B represents the modified pEGFP-C1 plasmid after insertion of the D2J derived RSS. Panel C shows flow data indicating that mutations in the N-terminal barrel region of GFP (represented by EGFP-Sac linker) do not affect GFP function after transfection of plasmid DNA into COS -7 cells. The construct used for EGFP-RSS flow data is shown in panel B.

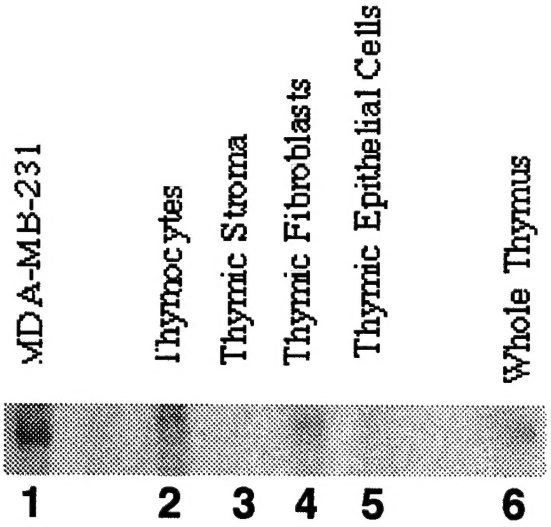


Figure 4. BRCA2 RNA is detectable in whole thymus and in purified thymocytes but not in thymic stroma. Brca2 levels were detected by Northern Blot using a BRCA2 specific probe. The MDA-MB-231 cell is used as a positive control. Thymic fibroblasts and epithelial cells are from cultured cell lines and may be differentialy activated compared to fibroblasts and epithelial cells from thymic stroma.

Acronym and Symbol Definition

ABMT	Ablative Bone Marrow Transplantation
CD44S	Standard form of CD44 containing exons 1-5, 15-17 and 18 or 19.
CD44v2-10	Isoform of CD44 containing exons 2 through exon 10.
CD44v8-10	Isoform of CD44 containing exons 8, 9, and 10.
EC-RF24	Primary human vascular endothelial cells immortalized with the E6/E7 genes of the human papilloma virus
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
GFP	Green Fluroescence Protein
HA	Hyaluronan
HDC	High Dose Chemotherapy
BMT	Bone Marrow Transplantation
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intracellular Adhesion Molecule
LPS	Lipopolysacchride
mAb	Monoclonal antibody
PBS	Phosphate-Buffered Saline
PdbU	Phorbol Ester
PVS	Perivascular Space
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RAG-1	Recombination Activating Gene 1
RAG-2	Recombination Activating Gene 2
RSS	Recognition Signal Sequence
TNF-α	Tumor Necrosis Factor-α

Statement of Work

Task 1: Months 1-6: Transfect recombination substrate, Rag-1 and Rag-2 into 293T cells, breast cancer cells or other appropriate cell lines and analyze rearrangement by flow cytometry and by RT-PCR. Optimize transfection efficiencies and ratios to maximize rearrangement of the recombination substrate.

Task 2: Months 3-9: Determine which BRCA2 sense and anitsense constructs or oligonucleotides to use which will increase or decrease BRCA2 expression in cultured cell lines by Northern Blot and/or Western Blot.

Task 3: Months 6-12 : Determine antisense oligonucleotides which decrease BRCA2 expression in primary thymocytes. Analysis of BRCA2 expression will be done by Northern and /or RT-PCR.

Task 3: Months 9-15: Determine the effects of under or over-expression of BRCA2 on T-cell receptor rearrangement using the *in vitro* recombination assay for cultured cell lines.

Task 4: Months 15-24: Determine the effects of under or over expression of BRCA2 on DNA repair processes using LM-PCR for primary thymocytes.

Task 5: Months 12-24: Confirm and quantitate the expression of BRCA2 in immature (unrearranged) vs mature (rearranged) thymocytes using competitive PCR.